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Award Number: DAMD17-01-1-0661

TITLE: Functional Characterization of Primary Mammary Cells with Life Span Extension Using Ectopic Telomerase

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REPORT DATE: August 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2003	Final (1 Aug 2001 - 31 Jul 2003)

4. TITLE AND SUBTITLE
Functional Characterization of Primary Mammary Cells with Life Span Extension Using Ectopic Telomerase

5. FUNDING NUMBERS
DAMD17-01-1-0661

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Abstract not provided

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
25

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Elmore et al, 2002 – Cancer Biology and Therapy
Elmore et al, 2002 – Journal of Biological Chemistry

Introduction

Telomere erosion and dysfunction have become hallmarks of senescent cells and contribute to the overall decline in proliferative ability. Direct experimental evidence shows that telomere shortening is at least partially responsible for the onset of cellular senescence. Over-expression of human telomerase, specifically the reverse transcriptase subunit hTERT, in normal human cells results in activation of telomerase, maintenance of telomeres, and extension of cellular life span (Bodnar et al., 1998). Importantly, even though telomerase is associated with nearly 90% of all human cancers, exogenous expression of telomerase allows normal cells to grow continuously without signs of cancer-associated changes (Morales et al., 1999), meaning that telomerase alone is not oncogenic. Our goal for this project has been to compare gene expression profiles in normal mammary epithelial (HME) cells with and without ectopic telomerase and define the changes in gene expression patterns that could distinguish young diploid cells from those that have an extended life span with telomerase. The idea has been to show that hTERT-expressing HME cells are more similar to young diploid cells than to immortalized HME cells. It is important to make a distinction between immortalized cells, which require significant gene expression changes, and our normal cells with telomerase, which, while continuously dividing, are not immortal in the classical sense. Our hypothesis is that ectopic telomerase provides primary mammary cells with genomic stability, preventing changes in gene expression typically associated with progression toward a breast cancer-like state. Therefore, our goal was to generate normal cell lines with and without telomerase expression and determine if telomerase acts as an aging/tumor suppressor by determining the consequences of exogenous telomerase expression in normal human mammary epithelial (HME) cells.

Body

Objective: To define the changes/differences in gene expression patterns between normal, diploid mammary epithelial cells, both young and approaching senescence, telomerase-expressing mammary cells, and spontaneously immortalized mammary lines.

Human mammary epithelial (HME) cells from a patient with Li Fraumeni syndrome (LFS) were isloated and cultured as previously described (Shay et al., 1995). HME 50-5 cells (a clone derived from LFS HME 50, which will be referred to as "50-5") harbor a germline mutation in a single allele of *p53*. Mortal 50-5 cells were stably infected with a retroviral vector encoding hTERT (50-5-hTERT), the catalytic component of human telomerase. As shown in Figure 1, we have successfully been able to generate 50-5 cell lines for over-expression of telomerase. Cells with telomerase activity (indicated by the 6bp laddering effect observed in lanes 4-12) showed elongated telomeres (right panel, Fig 1), and an extended cellular life span without observable senescence as indicated by staining with the senescence marker, β-galactosidase (not shown).

These 50-5 cell strain is able to spontaneously immortalize in culture with a dramatic increase in aneuploidy and reactivation of endogenous telomerase (Fig 1, left panel; Shay et al., 1995). Our stable telomerase-expressing 50-5 lines, as with other hTERT-expressing normal cells (Morales et al., 1999), have a stable karyotype and no reactivation of endogenous hTERT (not shown; **Elmore et al., 2002**). We show that over-expression of human telomerase in normal cells prevents the genomic instability associated with immortalization along with preventing the spontaneous immortalization event altogether. These data suggest that telomerase expressed in

normal human cells provides a protective mechanism involving telomere maintenance and gernoic stability. This is the first report that suggests that telomerase, although associated with most human cancers, can act in a tumor suppressive fashion by preventing genomic instability and the inevitable immortalization (**Elmore et al., 2002**).

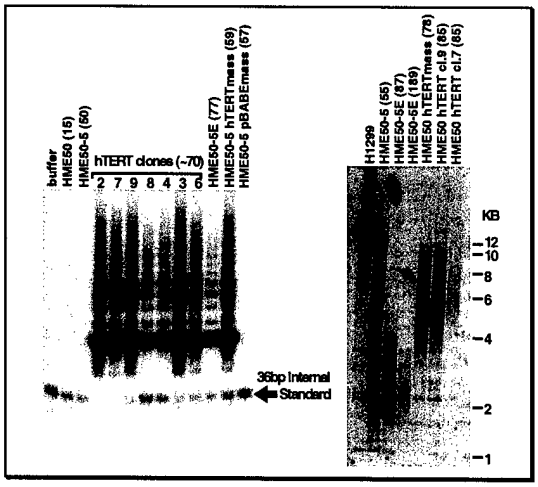

Figure 1. Stable expression of hTERT in HME cells. Left Panel: Stable 50-5 mass cultures and single cell-derived clones ectopically expressing hTERT and telomerase activity. Cultures were obtained by retroviral infection of hTERT, selected, and analyzed for telomerase activity using the TRAP assay. The 36-bp internal standard serves as a control for quantitation of activity. In all cases, 2000 cell equivalents were assayed. Right panel: Maintenance of telomere lengths in stable 50-5 mass cultures and clones expressing exogenous telomerase. hTERT-infected 50-5 cells maintained telomeres at a length of ~6.5kb. The telomeres of the uninfected, parental 50-5 cells were shorter (median TRF of ~4.2kb) and more heterogeneous in length, while 50-5E spontaneously immortalized cells maintained their telomeres at ~2.5kb. H1299, a human lung cancer cell line with unusually long telomeres, was included as a control. Values in parentheses represent the population doubling at the time of isolation.

We have analyzed 4 different cell types: young 50-5 cells, near senescent 50-5 cells, 50-5-hTERT cells, and immortalized 50-5E cells. We have found in our initial data analysis that the hTERT cells and the 50-5E cells are remarkably similar to each other, while the near senescent cells are somewhat dissimilar (Figure 2). This is likely due to the fact that both cell type, although not alike phenotypically, are constantly dividing, while the near senescent 50-5 cells are likely not. Most of the genes involved in the senescence program are related to the IGF-family of binding proteins (IGF-2 binding protein 3 and IGF-4 binding protein 4 are both down-regulated in near senescent cells compared to hTERT HMEs) and the cathepsin family member proteins, which are known to have increased expression with aging. In addition, we have found that p57 (kip2) is upregulated as normal cells approach senescence and is absent in the spontaneously immortal 50-5E, suggesting a p53-independent p57 role in aging and senescence.

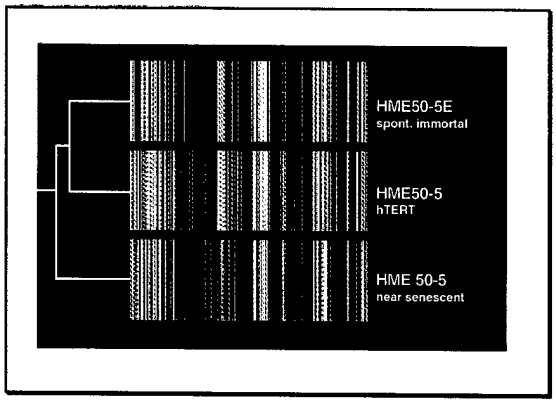

Figure 2. Cluster analysis of HME cell lines reveals similarities between the spontaneously immortal HME50-5E and the HME50-5-hTERT cells. Individual lines in the cluster analysis using the Affymetrix gene chips (HG_U95Av2) revealed that many proliferative genes were commonly expressed in those lines that were actively dividing (HME50-5E and HME50-5-hTERT) compared to the HME cells that were near senescent (HME50-5). More cluster analysis with a second round of hybridizations is forthcoming using young, diploid HME populations.

Using another direct comparison with the Affymetrix arrays, we compared the actively dividing HME 50-5 preimmortal cells to the 3 other cell lines. Figure 3 shows that there is little similarity

between any of the cell types tested, although faint clustering can be observed with the hTERT cells and the near senescent one, as well as between the young diploid HMEs and the spontaneously immortal one. Very little information can be drawn from these experiments in terms of clustering, but there are some interesting similarities and differences between the cell lines and strains that bear discussion.

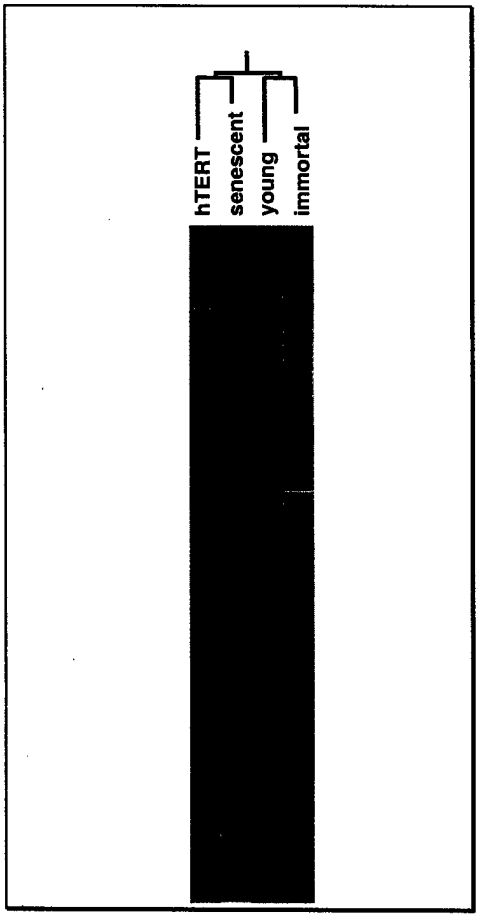

Figure 3. Cluster analysis of HME cell lines and strains shows very little similarity overall for any of the tested cell types. Using the Affymetrix gene chips (HG_U95Av2), each cell type was tested for gene expression patterns as before, and a cluster analysis was done. Addition of the young, diploid HME 50 cells shows little similarity to the HME50-hTERT line, as we expected based on the observed phenotype. Interestingly, we do find some areas similarity between the hTERT-expressing cells and the senescent, while the young, diploid HMEs reveal some, albeit faint, similarity to the immortal HMEs.

Tables I and II show changes observed using the Affymetrix arrays. Interestingly, in the hTERT-expressing cells, we observe a decline in protease inhibitors and an increase in serine protease expression. These date suggest that there may be some feedback inhibition of transcriptional expression of serine proteases as a result of a knockdown in protease inhibitor expression. However, as with all microarray data, biological confirmation using standard molecular and cellular techniques will be critical for understanding these types of regulatory pathways and how telomerase activation and telomere maintenance play roles in these processes. It is perhaps counterintuitive as to how over-expression of hTERT in HME cells results in an alteration in serine protease upregulation and/or protease inhibitor down-regulation. Even so, we have only just begun to elucidate the mechanisms affected by telomerase's ability to protect telomeres from erosion and dysfunction, which results in maintenance of genomic stability. Our experimental model (**Elmore et al. 2002**) suggests that telomere maintenance in Li Fraumeni cells results in prevention of both genomic instability and spontaneous immortalization, and it is likely that there are a number of other mechanisms that contribute to this cellular phenotype.

Table I. HME 50-hTERT cells have high levels of serine proteases and low levels of protease inhibitors

Gene	Young	Senescent	hTERT-infected	Spontaneously Immortal
Serine protease 3	92*	96	**609**	77
Serine protease 4	124	119	**983**	113
Cathepsin V/U	513	2786	**553**	52
Ser/Cys protease inhibitor, clade A	205	244	**Absent**	203
Human squamous cell carcinoma antigen (SCCA2)	72	241	**17**	Absent
SCCA2b	Absent	121	**Absent**	Absent
Tissue inhibitor of metalloproteinase 2 (TIMP2)	Absent	260	**Absent**	Absent
Kazal type 1 serine protease inhibitor	67	93	**Absent**	Absent

*Values represent raw Affymetrix Data

One of our goals in initiating this project was to define additional senescence-associated genes, perhaps those that are breast-specific. In addition to the protease data, we have identified nearly 10 novel genes that may be associated with mammary epithelial cell senescence and many of these targets (9 out of 10) were found in a completely independent experiment. These are targets are currently being followed for changes in expression at the RNA and protein levels, and additional biological experiments (knock down with siRNA) are in progress.

Table II. Near senescent HME 50 cells selectively express age-associated genes

Gene	Young	Senescent	hTERT-infected	Spontaneously immortal
P57/KIP2	71*	**212**	24	70
Kallikrein 6 (neurosin)	Absent	**149**	Absent	Absent
Small proline-rich protein 3 (SPRR3)	Absent	**221**	Absent	Absent
Sciellin (SCEL)	49	**329**	58	Absent
Cathepsin V/U	513	**3141**	553	52
PTB domain adaptor protein (CED-6)	47	**213**	18	56
Growth arrest-specific 3 protein (GAS3)	67	**266**	Absent	58
TIMP2	Absent	**225**	Absent	Absent
Latexin protein (LXN)	Absent	**205**	Absent	Absent
TRIM 2	214	**524**	16	45
Keratin 16	1342	**2987**	407	670
Homo sapien B-factor (properdin [BF])	Absent	**327**	Absent	Absent

Candidates for new senescence-associated genes are also listed (in italics).
*Values represent raw Affymetrix Data

We have expanded the study to include the differences between breast tumor cells with and without exogenous hTERT after treatment with the chemotherapeutic compound, Adriamycin. Importantly, MCF-7 cells undergo a senescence program after treatment, making the comparison to untreated cells along the lines of normal cells in the proliferative pool versus those approaching senescence. This type of characterization will provide us with important targets related not only to those genes important in Adriamycin-induced senescence but also those that may be necessary to replicative senescence observed in normal mammary epithelial cells. Table 1 shows the important cell cycle and p53-related gene expression differences. We are currently following many of these targets for changes that occur after treatment with other modalities.

Comparative Expression Profiles of Select Targets

	Gene	MCF-7+AdR/ Untreated	MCF-7 neo+AdR/ Untreated	MCF-7 E6+AdR/ Untreated
cell cycle regulators	Cyclin A	10x ↓	3.5x ↓	↑
	Cyclin B	10x ↓	4.8x ↓	↑
	Cyclin E2	10x ↓	absent	↑
	Cyclin F	5.0x ↓	absent	NC(p)
	PCNA	5.0x ↓	2.0x ↓	↑
	cdc-2	6.7x ↓	NC(a)	NC(p)
	TGF-β	14x ↑	26x ↑	1.6x ↓
p53-related	p21 (waf-1)	12x ↑	↑	absent
	GADD45	4.2x ↑	↑	NC(a)
	Bax-α	3.1x ↑	1.9x ↑	absent
	Bax-γ	3.0x ↑	2.3x ↑	absent
	p53	NC(p)	NC(p)	NC(p)
decreased in MCF-7 E6 cells	IGFBP-5	14x ↑	12x ↑	absent
	JAK-1	4.1x ↑	↑	absent
	MRP-3B	↑	3.2x ↑	absent
	c-jun	12x ↑	↑	absent
	BID	4.1x ↑	1.6x ↑	absent
	BIK	↑	↑	absent
	ERF-1	3.2x ↑	11x ↑	6.2x ↓

[confirmation of the following targets was accomplished using the OpArrays from Operon Technologies: cyclins B and E, PCNA, p21, GADD45, c-jun, p53, and superoxide dismutase]

Our goal is not to characterize every gene that is differentially expressed, but to follow important genes related to DNA damage, telomere maintenance, and the senescence program in both the HME model system and the MCF-7 breast tumor cell system. Understanding the role of these genes will provide important data for defining the mechanisms of telomere-related and stress-induced senescence, as well as providing critical preliminary data for future DOD or NIH proposal in breast cancer biology.

Key Research Accomplishments

1-telomerase-expressing normal mammary epithelial cells are molecularly distinct from normal mammary cells approaching senescence

2-expression of genes related to proliferation/cell cycle regulation are commonly expressed in immortal mammary cell lines and the matched normal cell strains with etopic telomerase expressed

3-further experiments on intimately comparing the gene expression profiles of young mammary cells with those with telomerase over-expression are underway, but preliminary data suggests few changes in expression patterns

Recommended Changes to the Proposed Work Based on Additional Findings

We were granted a no-cost extension of the 1-year Concept Award to finish the proposed experiments for reasons related to personnel and the fact that the microarrays from Operon are no longer sold due to licensing and patent problems with Affymetrix, as of July 2001. Because of this, we have had to utilize Affymetrix arrays and directly compare to the few OpArrays we currently have on hand. We have expanded our proposed research to looking at breast tumor cell lines with and without telomerase over-expressed (MCF-7 and MCF-7-hTERT) for gene expression changes before and after treatment with either Adriamycin or γ-irradiation, as well as the differences in mammary cells with and without telomerase after treatment. This will provide us with important molecular targets for defining the mechanisms of Adriamycin-induced and γ-IR-induced DNA damage in both tumor and normal cells, which will be useful for future grant applications.

Reportable Outcomes

Manuscripts

Elmore,L.W., K.C.Turner, L.S.Gollahon, M.R.Landon, C.K.Jackson-Cook, and S.E.Holt. 2002. Telomerase protects cancer-prone cells from chromosomal instability and spontaneous immortalization. Cancer Biology and Therapy 1:395-401.

Elmore,L.W., C.W. Rehder, M.Di, P.A.McChesney, C.K.Jackson-Cook, D.A.Gewirtz, and S.E.Holt. 2002. Adriamycin-induced replicative senescence in tumor cells requires functional p53 and telomere dysfunction. J. Biol. Chem. 277:35509-35515.

Abstracts/Presentations

Elmore,L.W., C.W.Rehder, X.Di, C.K.Jackson-Cook, D.A.Gewirtz, and S.E.Holt. Cold Spring Harbor meeting, Telomeres and Telomerase. May 2003.

Elmore,L.W., K.C.Turner, C.W.Rehder, L.S.Gollahon, M.R.Landon, C.K.Jackson-Cook, and S.E.Holt. AACR: The role of telomeres and telomerase in cancer, San Francisco, CA. December 2002.

Holt,S.E., L.W.Elmore, C.W.Rehder, X.Di, P.A.McChesney, C.Jackson-Cook, and D.A.Gewirtz. Molecular Genetics of Aging, Cold Spring Harbor, NY. October 2002.

Holt,S.E., C.I.Dumur, A.Ferreira-Gonzalez, D.A.Gewirtz, and L.W.Elmore. DOD Breast Cancer Research Meeting, Orlando, FL September 2002.

Holt,S.E., L.W.Elmore, Y-M.Di, A.Akalin, P.A.McChesney, and D.A.Gewirtz,. DOD Breast Cancer Research Meeting, Orlando, FL September 2002.

Invited Seminars

Holt,S.E. Mini-Medical School, Science Museum of Virginia, Richmond, VA. March 2003.

Holt,S.E. Department of Neurooncology, MCV/VCU, Richmond, VA. March 2003.

Holt,S.E. Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2003.

Holt,S.E. Bridges Program, Virginia State University, Petersburg, VA. November 2002.
Holt,S.E. Molecular Biology and Genetics Program, MCV/VCU, Richmond, VA. October 2002.
Holt,S.E. Department of Pharmacology/Toxicology, MCV/VCU, Richmond, VA October 2002.

Development of Cell Lines

We have developed cell lines for telomerase over-expression in HME31, HME50, and MCF-7, as well as MCF-7 cells expressing the human papillomavirus type 16 E6 protein for elimination of p53 function.

Funding Applied For

Department of Defense Breast Cancer Research Program, IDEA award, May 2003
Department of Defense Breast Cancer Research Program, Predoctoral Traineeship award, May 2003
National Institutes of Health, R-01 mechanism, June 2003
The Susan Komen Foundation, Postdoctoral Fellowship, August 2003

Conclusions

Having established a model system for studying the effects of telomerase over-expression in normal mammary cells, we have utilized Affymetrix microarray technology to analyze the gene expression patterns and how extended lifespan with exogenous telomerase will alter expression. Thus far, this work has identified potential IGF-related and cathepsin family proteins that appear associated with senescence, as well as a single tumor suppressor gene that may also be associated with p53-independent senescence in our Li Fraumeni mammary epithelial cell model system. Our more recent data suggests a number of potentially novel senescence-associated genes that may be specific for mammary cells. As with all microarray analyses, any discovered alterations in gene expression will require significant molecular and cellular follow-up experimentation to biologically confirm the findings. Our plan is to expand this survey to a more thorough analysis of the Affymetrix data and begin the rigorous proof of these alterations. Our goal is to define changes in gene expression patterns related to telomere-induced or stress-induced senescence in normal mammary and breast tumor cells, as well as determine if long-term expression of telomerase activity in normal mammary cells alters gene expression patterns when compared to normal young HME cells.

References (**bold** indicates PI authored papers)

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu C-P, Morin GB et al. 1998. Extension of life-span by introduction of telomerase in normal human cells. Science; 279: 349-352.

Elmore LW, Turner KC, Gollahon LS, Landon MR, Jackson-Cook CK, and Holt SE. 2002. Telomerase protects cancer-prone cells from chromosomal instability and spontaneous immortalization. Cancer Biology and Therapy; 1:395-401.

Morales CP, Holt SE, Ouellette M, Kaur K.J, Yan Y, Wilson KS et al. 1999 Absence of cancer associated changes in human fibroblasts immortalized with telomerase. Nat Genet; 21: 115-118.

Shay JW, Tomlinson G, Piatyszek MA, Gollahon LS 1995 Spontaneous *in vitro* immortalization of breast epithelial cells from a patient with Li-Fraumeni syndrome. Mol Cell Biol; 15:425-432.

APPENDIX COVER SHEET

[Cancer Biology & Therapy 1:4, 391-397, July/August 2002]; ©2002 Landes Bioscience

Research Article

Telomerase Protects Cancer-Prone Human Cells from Chromosomal Instability and Spontaneous Immortalization

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Received 3/13/02; Accepted 4/5/02

Previously published online as a CB&T "Paper in Press" at:
http://landesbioscience.com/journals/cancerbio/papersinpress/inpress14.html

KEY WORDS

Cancer, Immortality, Senescence, Telomerase, Telomere

ABBREVIATIONS:

DAB	Diaminobenzidine
HME	Human mammary epithelial
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA component
LFS	Li Fraumeni Syndrome
M1	Mortality Stage 1
M2	Mortality Stage 2
PD	Population doubling
PBS	Phosphate buffered saline
SKY	Spectral karyotyping
TRAP	Telomeric Repeat Amplification Protocol
TRF	Telomeric Restriction Fragment

ABSTRACT

Studies were conducted to directly test whether the introduction of telomerase protects cancer-prone human mammary epithelial cells from chromosomal instability and spontaneous immortalization. Using a model for Li Fraumeni Syndrome (LFS), infection of human telomerase resulted in maintenance of telomere lengths, extension of in vitro lifespan, and prevention of spontaneous immortalization. In stark contrast to the spontaneously immortalized LFS cells, cells expressing ectopic telomerase displayed a remarkably stable karyotype and even after >150 population doublings, did not express endogenous telomerase. Since the hTERT-infected and spontaneously immortal LFS cells, like the parental cells, exhibit loss of p53 function, our data suggests that telomere shortening is the primary driving force for the genomic instability characteristic of LFS cells, while p53 inactivation is necessary for triggering the spontaneous immortalization event. Collectively, our data indicate that exogenous telomerase prevents chromosomal instability and spontaneous immortalization of LFS cells, suggesting a unique protective role for telomerase in the progression to immortalization.

INTRODUCTION

The immortalization of human cells is a critical step in tumorigenesis. "Classical" immortality requires cells to bypass cellular senescence (M1), attain other mutations in the extended life span, and ultimately overcome crisis (M2).[1] The onset of cellular senescence appears to be triggered by telomere shortening, which is caused by the "end replication problem" imposed during the synthesis of linear chromosomes.[2-5] If cells bypass senescence and remain proliferative, telomeres continue to shorten until they reach a critically short length and undergo crisis. To overcome crisis, cells need a telomere maintenance mechanism.

The ribonucleoprotein, telomerase, provides the necessary enzymatic activity to restore telomere length in >85% of human cancers.[6-8] However, while telomerase is necessary for immortalization and essential for continued cancer cell proliferation, expression of exogenous telomerase in normal human cells appears to prevent cellular senescence without phenotypic features indicative of malignant transformation.[9-10] In this study, we directly test whether exogenous telomerase is also able to protect cancer-prone human cells from genomic instability and activation of endogenous telomerase, hallmarks of "classical" immortalization.[11] We show that immortal cells are biologically distinct from those cells that have an extension of lifespan via ectopic telomerase expression. While some ectopic telomerase-expressing normal cells have the ability to continuously divide, they do not bypass senescence; senescence is prevented altogether without acquisition of the requisite mutations leading to crisis and immortalization. Using a model for Li-Fraumeni Syndrome (LFS),[12] we find that exogenous telomerase protects LFS human mammary epithelial (HME) cells from spontaneous immortalization, and that the chromosomal instability characteristic of LFS cells[12-15] is primarily dependent upon telomere erosion. Our results conclusively show that introduction of telomerase prevents spontaneous "classical" immortalization, providing the first experimental evidence that telomere erosion is the driving force for the genomic instability required for classical immortalization. In addition, our data in human Li Fraumeni cells suggest that lack of p53 function only serves to facilitate the "classical" immortalization process in cells with telomere dysfunction.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Retroviral Infections. 50-5 cells were obtained from normal breast tissue of a 31-year-old Li-Fraumeni patient undergoing surgery for breast cancer.[12] Monolayer cultures were maintained in a chemically-defined media (SF170),[12] which was changed every 2-3 days. The breast epithelial cells obtained from this patient spontaneously immortalize, albeit at a low frequency.[12] 50-5E represents one of four clones, which, following a period of crisis,

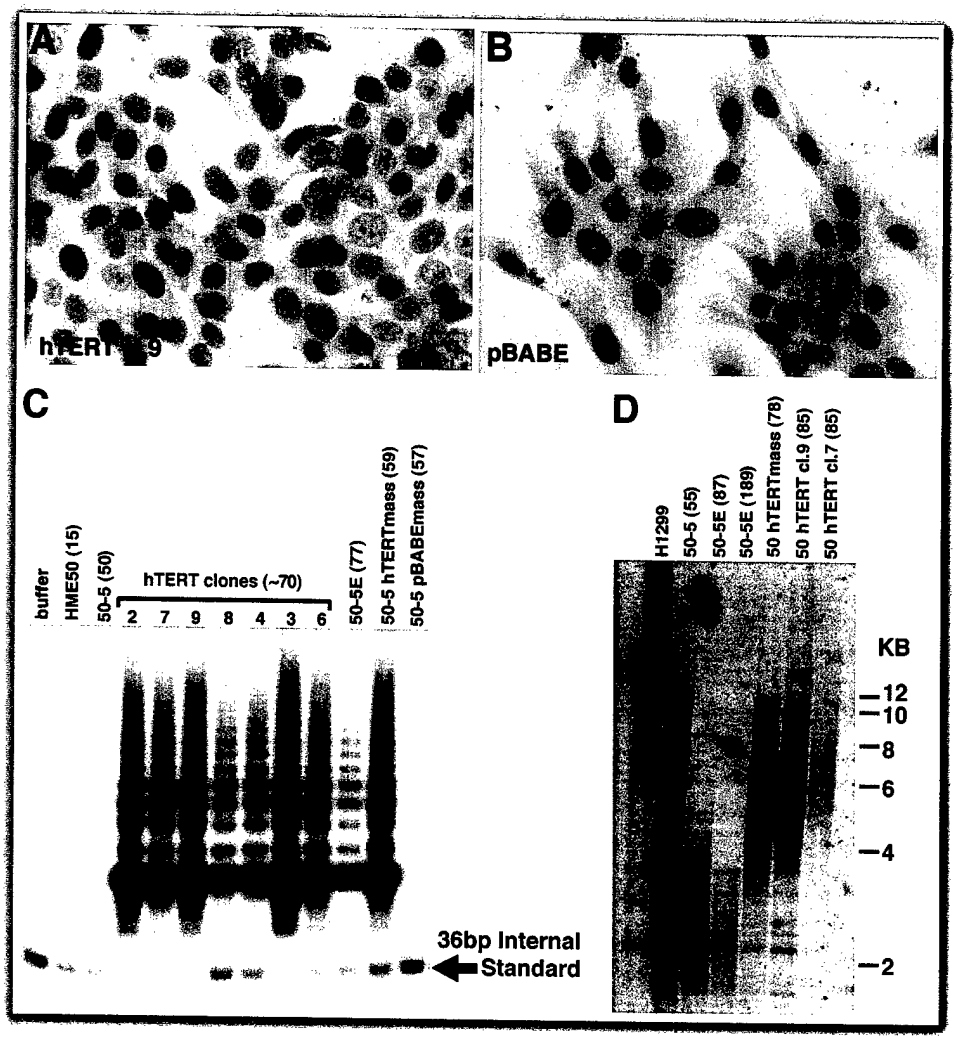

Figure 1. Ectopic hTERT expression localizes to the nucleus and is sufficient to reconstitute telomerase activity and maintain telomere lengths in LFS 50-5 cells. (A) Intense nuclear staining of hTERT in a representative 50-5 clone (cl. 9; PD 105) expressing exogenous telomerase. Original magnification 20x. (B) Undetectable hTERT immunocytochemical staining in pBABE mass-infected 50-5 cells (PD 58). Original magnification 20x. (C) Stable 50-5 mass cultures and single cell-derived clones ectopically expressing hTERT and telomerase activity. Cultures were obtained by retroviral infection of pBABE-hTERT, selected with puromycin, and analyzed for telomerase activity by TRAP as described in "Materials and Methods". The 36-bp internal standard serves as a control for quantitation of activity. In all cases, 2000 cell equivalents were assayed. Numbers in parentheses indicate the population doubling at the time of cell lysing. (D) Maintenance of telomere lengths in stable 50-5 mass cultures and clones expressing exogenous telomerase. hTERT-infected 50-5 cells maintained telomeres at a length of ~6.5kb. The telomeres of the uninfected, parental 50-5 cells were shorter (median TRF of ~4.2kb) and more heterogeneous in length, while 50-5E spontaneously immortalized cells maintained their telomeres at ~2.5kb. H1299, a human lung cancer cell line with unusually long telomeres, was included as a control. Values in parentheses represent the population doubling at the time of DNA isolation.

spontaneously immortalized, as characterized by activation of endogenous telomerase and continuous cell growth. Retroviral vectors consisted of the parental vector (pBABE-puro) or vector containing the coding sequence for hTERT (pBABE-hTERT), the catalytic component of human telomerase. Retroviral stocks, prepared as described previously,[16] were used to infect mortal (telomerase-negative), 50-5 cells at population doubling (PD) 45. Following puromycin selection (400ng/ml) for 4 days, a stable mass population of 50-5 cells expressing ectopic telomerase was established. In order to obtain single cell-derived clones, mass-infected cells were seeded at 100 cells per 100-mm dish, and then 14 days later expanded using sterile cloning cylinders. Infection of the empty vector (pBABE-puro) as well as uninfected 50-5 cells served as negative controls.

MCF-7 (provided by Dr. David Gewirtz, The Medical College of Virginia at Virginia Commonwealth University, Richmond, VA), a human breast cancer cell line with wild type p53, was cultured in RPMI + 10% fetal bovine serum and served as a positive control for p53 and $p21^{waf-1}$ Western blotting. H1299, a human lung carcinoma cells line, was cultured in DMEM+ 10% fetal bovine serum for the purpose of isolating genomic DNA as a control for the Telomeric Restriction Fragment (TRF) assay.

Immunocytochemistry. Cells were seeded onto 8-well chamber slides at 4 x 10^4 cells per well (Nalgene Nunc, Naperville, IL). Between 24-48 hours after seeding, the slides were rinsed briefly in a phosphate buffered saline (PBS), pH 7.4, and then fixed for 10 minutes at room temperature in acetone. The cells were rehydrated in PBS and then incubated in 0.3% hydrogen peroxide diluted in PBS for 10 minutes to quench endogenous peroxidase activity. Following a 15-minute blocking step in normal goat serum (1:50 dilution in PBS), either 1:500 diluted hTERT monoclonal antibody (Geron Corporation, Menlo Park, CA) or PBS (negative control) was applied to each well for overnight incubation at 4°C in a humidifier chamber. Antigen-antibody complexes were visualized using a streptavidin-biotin staining technique (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations. Diaminobenzidene (DAB) was used as a chromogen, and hematoxylin served as a counterstain.

Telomeric Repeat Amplification Protocol (TRAP). Telomerase activity was determined by TRAP as previously described[17] with minor modifications. The TRAPeze telomerase detection kit (Intergen, Gaithersburg, MD), that includes a 36-bp internal standard to allow quantification of activity, was used.[18] Following telomerase extension for 30 minutes at room temperature, extended products were amplified by a two-step PCR (94°C for 30 seconds, 60°C for 30 seconds) for 27 cycles. Products were separated by polyacrylamide gel electrophoresis and then exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA). Quantitative estimates of telomerase activity were calculated by determining the ratio of 36-bp internal standard to the 6-bp telomerase-specific ladder using ImageQuant software.[18]

Telomeric Restriction Fragment (TRF) Length Measurements. High molecular weight genomic DNA was isolated using the Qiagen Genomic-tip System (Qiagen, Valencia, CA) according to the manufacturer's instructions. TRF length measurements were performed as described elsewhere.[2,19-20] The median TRF length was estimated at the peak position of the hybridization signal based on electrophoresis of a radiolabeled 1Kb ladder (Gibco-BRL, Rockville, MD).

RT-PCR Analysis of Endogenous vs. Exogenous hTERT Expression. Total RNA was isolated from cells in log growth phase using Trizol (Gibco-BRL, Rockville, MD) as recommended by the manufacturer. Three µg of total RNA from each cell culture was reverse transcribed in a 20µl reaction volume using decamers and the regular reaction buffer provided in the first strand synthesis kit, RETROscript (Ambion Inc.) and according to the manufacturer's established protocol. A 2.5 µl aliquot of cDNA was used for PCR amplifications, also following the instructions provided in the RETROscript kit. hTERT was amplified using the oligonucleotide primer hTERT (5'-GACTCGACACCGTGTTCACCTAC-3') paired with either endo #1 (5'-ACGTAGAGCCCGGCGTGACAG-3') or pBABE (5'-GACA-

CACATTCCACAGGTCG-3'),[21] which selectively amplify endogenous or exogenous hTERT, respectively. For both primer pairs, the thermocycling conditions consisted of an initial heating at 94°C for 5 minutes, followed by a total of 34 cycles of 94°C for 45 cycles, 62°C for 30 seconds and 72°C for 45 seconds. The control amplification of 18S RNA was performed using 18S PCR primer pairs (Ambion, Inc) with the same thermocycling conditions with the modification that only 22 cycles were completed. Amplified products (exogenous hTERT: 175-bp; endogenous hTERT: 219-bp; 18S: 488-bp) were resolved on a 2% agarose gel and visualized by staining with ethidium bromide.

Chromosomal Studies. To monitor chromosomal instability, metaphase spreads were analyzed using both GTG-banding and spectral karyotyping (SKY). For GTG-banding, metaphase chromosomes were harvested and analyzed using standard methods,[22] with both consistent and incidental chromosomal aberrations being noted. SKY was performed according to the manufacturer's protocol (Applied Spectral Imaging Inc., Carlsbad, CA) as described previously.[23] At least 20 metaphase spreads were analyzed for each cell line. The frequency of structural chromosomal abnormalities was compared using a Pearson's chi square test (SAS Institute, Inc.). Photographic documentation was done using either a Cytovision Image Analysis System (GTG-banding; Applied Imaging) or an Applied Spectral Imaging System (SKY).

Immunoprecipitation and Western Blotting Analyses. For immunoprecipitation, cells were rinsed in PBS and then placed for 1 hour on ice in lysis buffer (150 mM NaCl, 0.5% Nonidet-P40, 5 mM EDTA, 20 mM Tris-HCL [pH 8.0]) and a protease inhibitor cocktail (Sigma Chemical Company, St. Louis MO). Following lysate clarification for 10 minutes at 13,000 rpm, 3 x 10^5 cell equivalents were immunoprecipitated with 1 µg of either a wild type-specific (Ab-5; Oncogene Research Products, Boston, MA); a mutant-specific (Ab-3; Oncogene Research Products), or a pantropic (Ab-6; Oncogene Research Products) p53 monoclonal antibody overnight at 4°C. Antigen-antibody complexes were bound to Protein G Agarose (Roche Diagnostics, Indianapolis, IN), washed three times in lysis buffer, and then separated by SDS-PAGE. Following the electrotransfer of proteins onto Hybond ECL nitrocellulose membrane (Amersham, Arlington Heights, IL), a standard Western blotting protocol was performed. Briefly, the membrane was blocked for 1 hour in 5% dry milk in PBS +0.01% Tween-20, and then incubated with CM-1 p53 polyclonal antibody (1:750 dilution; Vector Laboratories) for 1 hour followed by incubation with horseradish peroxidase-labeled goat anti-rabbit IgG (1:5000; Vector Laboratories) for 45 minutes. Four 10-minute washes in PBS/Triton were performed after treatments with the primary and secondary antibodies. A luminol-based chemiluminescent reaction (ECL Reagent, Amersham) was used for detection of product.

For the p53 and $p21^{waf-1}$ Western blot, the various cell cultures were lysed in a standard RIPA buffer (50mM Tris [pH 7.4], 150 mM NaCl, 1.0% Triton X-100, 0.1% SDS, 100 mM dithiothreitol, and a cocktail of protease inhibitors) four hours after a single dose of γ-irradiation (10Gy). Protein concentrations were determined using a Lowry-based spectrophormetric assay (BioRad, Hercules, CA), according to the manufacturer's protocol. Twenty µg of each sample were separated by SDS-PAGE and electrotransferred onto nitrocellulose membrane. Blotting was performed as described above using the pantropic p53 antibody (1:500) and a $p21^{waf-1}$ monoclonal (1:500; Signal Transduction Laboratories/Pharmingen, San Diego, CA), followed by peroxidase-conjugated anti-mouse IgG (1:10,000; Amersham). To control for protein loading, the membrane was subsequently probed with an actin antibody (1:500; Sigma Chemical Company).

RESULTS

Ectopic hTERT is Sufficient to Reconstitute Telomerase Activity and Maintain Telomere Lengths in LFS Cells. In order to test whether ectopic telomerase protects cancer-prone cells from chromosomal instability and spontaneous immortalization, we have utilized a LFS HME culture system. HME 50-5 cells (a clone derived from LFS HME 50, which will be referred to as "50-5") harbor a germline mutation in a single allele of p53.[12] Mortal

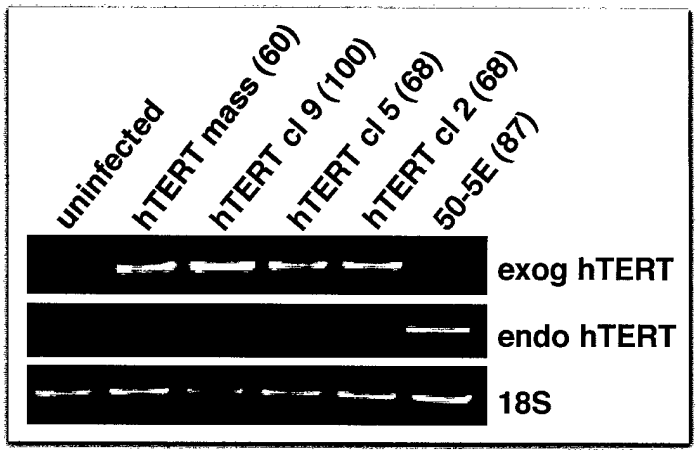

Figure 2. Ectoptic expression of hTERT in LFS 50-5 cells prevents activation of endogenous hTERT. Semi-quantitative RT-PCR was performed using primer sets, that specifically distinguish between exogenous and endogenous hTERT (Hahn et al., 1999).[21] Amplification of 18S was included as a control. The exogenous-specific primers amplified a 175-bp product, the endogenous-specific primers a 219-bp product, and the 18S primer set a 488-bp product. Values in parentheses represent the population doubling at the time of RNA isolation.

50-5 cells were stably infected with a retroviral vector encoding hTERT (50-5-hTERT), the catalytic component of human telomerase.[24-25] Immunocytochemical staining revealed that ectopic hTERT protein was localized exclusively to the nucleus (Fig, 1A), while immunostaining was undetectable in the pBABE-puro mass-infected controls (Fig. 1B). As reported previously,[26-27] strict nuclear localization is highly consistent with the role of telomerase in telomere maintenance. We were unable to visualize endogenous hTERT protein in either the 50-5E spontaneously immortalized cells[12] or a human breast cancer cell line (MCF-7) (data not shown), despite detectable telomerase activity (Fig. 1C and data not shown). Similarly, others have only been able to detect ectopic hTERT using this same monoclonal antibody (James Trager, Ph.D., Geron Corp., personal communication).

In agreement with findings in a variety of in vitro cell systems,[17,28-31] introduction of exogenous hTERT was sufficient to reconstitute telomerase activity in 50-5 cells (Figure 1C). We confirmed that stable, mass-infected hTERT cells and single cell-derived clones had substantial activity, while telomerase activity was undetectable in uninfected and pBABE-infected cells. A terminal restriction fragment (TRF) assay verified that ectopic telomerase in 50-5 cells resulted in maintenance of telomere lengths (Fig. 1D). Stable hTERT mass-infected and representative clones maintained telomere lengths at ~6.0 kb while the telomeres of uninfected cells were shorter (median telomere length of ~4.2 kb) and more heterogeneous in length. Spontaneously immortal 50-5E cells consistently maintained their telomeres at a length of about 2.5 kb.

Absence of Endogenous Telomerase Activation in LFS Cells Ectopically Expressing hTERT. One of the primary causes of cellular senescence is telomere shortening.[31-32] In order for a cell to continuously proliferate, it must first bypass senescence (i.e., through inactivation of tumor suppressors such as p53 and pRb) and then evade crisis by acquiring a telomere maintenance mechanism.[1] Consistent with this model, we show by semi-quantitative RT-PCR that spontaneously immortal 50-5E cells express endogenous hTERT (Fig. 2) and maintain telomeres, albeit at a relatively short length (see Fig. 1D). In contrast, those cells expressing ectopic telomerase activity (and hence, maintaining telomere lengths) did not express endogenous hTERT even after >50 PDs post-infection. To date, the 50-5-hTERT cells have been passaged >150 PDs (more than 100 PDs post-infection), and they remain contact inhibited and still do not express endogenous telomerase (data not shown), a finding that is intuitively not surprising since ectopic telomerase would obviate the need for an internal telomere maintenance mechanism. As expected, uninfected, mortal 50-5 cells, which have undetectable telomerase activity (Fig. 1C), did not express endogenous or exogenous hTERT (Fig. 2).

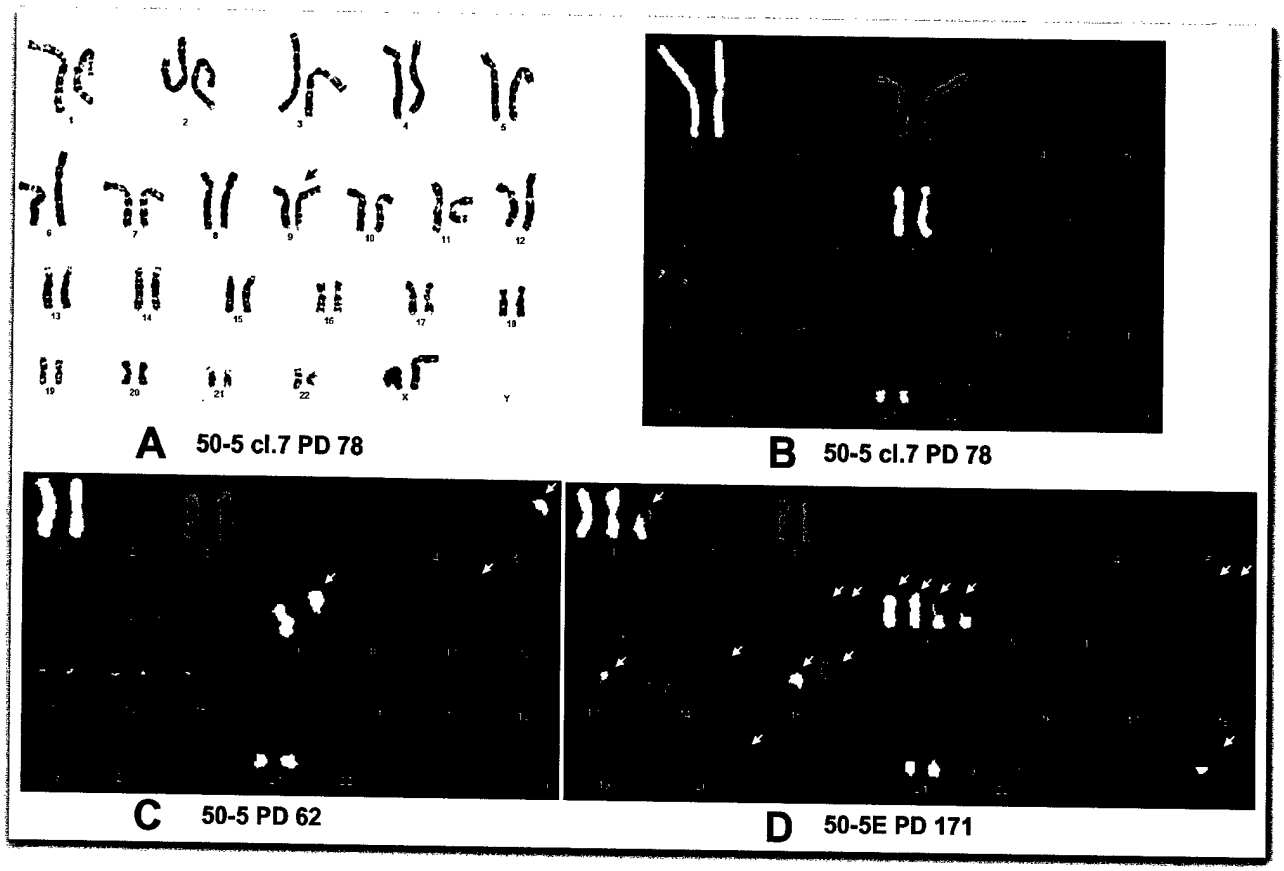

Figure 3. Reduction in chromosomal aberrations in hTERT-expressing LFS cells. Representative metaphase spreads are shown following standard GTG-banding (A) and SKY (B) [both metaphase spreads from clone 7 PD 78]. The GTG-banding patterns (A) show a pericentric inversion of chromosome 7, which is a normal variant that is not clinically significant but was present in all of the cell lines, thereby confirming their common cellular origin. Following SKY (B), all of the chromosomes from the exogenous hTERT cells showed a single color (indicating no interchromosomal rearrangements). On the structurally normal acrocentric chromosomes (chromosomes 13, 14, 15, 21, and 22) in frame B, the expected color variations were seen on the short arm regions, the latter of which result from the presence of heteromorphic, repetitive sequences that lack chromosome specificity. For the clonal lines expressing exogenous hTERT, the majority of the cells' chromosomal complements were within normal limits, while the 50-5 and the 50-5 E (PD 171) cells had multiple abnormalities (indicated by arrows), which tended to be different in each metaphase spread, demonstrating cytogenetic heterogeneity. (C) shows a metaphase spread from cell line 50-5 (PD 62) having 3 structural chromosomal abnormalities (white arrows) [der(5)t(5;9); der(9)t(9;11); der(11)t(6;11)] following SKY. In frame (D), a representative metaphase spread from the near tetraploid 50-5E line (PD 171) is shown (following analysis with SKY) that has 15 structurally abnormal chromosomes (white arrows) [der(X)ins(X;9); der(1)ins(1;20); der(8)t(X;8)x2; i(9)(q10)x2 (seen by reverse DAPI image [not shown]); der(9)t(7;9)x2; der(12)t(12;16)ins(16;8)x2; der(13)t(13;21); der(14)t(11;14); der(15)t(15;21); der(15)t(3;15); der(20)t(16;20)]. One could speculate that four of these abnormalities [the der(8)t(X;8); i(9)(q10); der(9)t(7;0) and the der(12)t(12;16)ins(16;8)] were present before the cell line became polyploid since these findings are present in duplicate (x2) copies.

Ectopic Telomerase Expression Confers Chromosomal Stability to LFS Cells. Besides activation of endogenous telomerase, genomic instability is another distinctive feature of cells that have bypassed senescence and crisis (i.e., "classically" immortalized cells). Recognizing that LFS cells are inherently prone to genetic alterations and subsequent immortalization,[12,15] we have used both GTG-banding and spectral karyotyping (SKY) to compare the chromosomal complements of hTERT-infected 50-5 clones with those of uninfected 50-5 cells and spontaneously immortalized 50-5E cells. The uninfected 50-5 cells, as well as hTERT-expressing clones, maintain predominantly a diploid karyotype, while the 50-5E cells were all near tetraploid, showing variability between cells in chromosomal numbers (Fig. 3 and Table 1). Although we show data for only a single spontaneously immortalized clone, all HME50 clones that went through crisis spontaneously were highly aneuploid and had substantial karyotypic abnormalities (data not shown). All metaphases contained a normal variant inversion of chromosome 9 [inv(9)(p11q12)], which is expected since they all have a common origin from 50-5 cells. In addition to having numerical abnormalities, a significant increase in structural chromosomal rearrangements was observed in the spontaneously immortalized 50-5E cells after 171 PDs (12.2 ± 3.3) when compared to the lower PD (4.1 ± 1.3) (χ^2_5=24.4, p<0.005), and to the 50-5 exogenous hTERT-expressing clones (clone 7: 0.1 + 0.4 in predominant diploid cells (χ^2_5=65.2, p<0.001); clone 9: 0.03 + 0.18 in diploid cells (PD 74) (χ^2_5=72.1, p<0.001), 0.6 ± 1.1 (PD 125) (χ^2_5=51.6, p<0.001) (Table 1). The types of aberrations seen included insertions, deletions, translocations (involving 2 and 3 chromosomes), and duplications. Also, the 50-5E and uninfected 50-5 cultures showed marked cytogenetic heterogeneity with several different single cell findings being noted between metaphases. In the case of high passage, uninfected cells (PD 62), the number of metaphases having incidental chromosomal anomalies was significantly higher than that observed in the uninfected 50-5 cells at PD 50 (Table 1) (χ^2_5=14.3, p<0.01), as well as exogenous hTERT clones [cl 7 PD 78 (χ^2_5=18.9, p<0.002); cl 9 PD 74 (χ^2_5=18.8, p<0.002); cl 9 PD 125 (χ^2_5=14.6, p<0.012)]. In contrast, the hTERT-expressing clones had very few consistent or single cell aberrations, even after many PDs (Table 1). Collectively, our cytogenetic data demonstrate that 50-5 cells ectopically expressing telomerase do not demonstrate the chromosomal instability characteristic of LFS 50-5 cells predisposed to spontaneous immortalization. Moreover, the remarkably stable karyotype of the 50-5-hTERT cells further supports their distinction from "classically" immortalized cells.

Telomerase-Expressing LFS Cells Express Wild Type and Mutant p53 Protein, Yet Display Undetectable p53 Transcriptional Activation Activity. Previous data indicate that several LFS-associated mutant p53 proteins, including the 133 mutant expressed in the 50-5 cells, exhibit transdominant negative effects on biochemical properties of wild type p53.[33] However,

Table 1 **STRUCTURAL CHROMOSOMAL ABNORMALITIES OBSERVED IN THE LFS HME CELL LINES**

Cell line	Number Metaphase Spreads Analyzed	Number Abnormalities per Metaphase (x ± s.d.)	Number Metaphase Having Consistent Anomalies[1] 0	1	2	3	4	5	≥6	Number Metaphase Having Incidental Anomalies[2] 0	1	2	3	4	5	≥6
50-5																
PD 50																
Near diploid	18	0.5 ± 0.5	15	3	0	0	0	0	0	11	6	1	0	0	0	0
Near tetraploid	6	1.7 ± 1.2	3	3	0	0	0	0	0	2	2	1	1	0	0	0
Total	24															
PD 62																
Near diploid	22	1.4 ± 1.7	22	0	0	0	0	0	0	**11**	**0**	**6**	**3**	**1**	**0**	**1**
Near tetraploid	6	0.3 ± 0.5	3	3	0	0	0	0	0	2	2	1	1	0	0	0
Total	26															
50-5 cl 7																
PD 78 (hTERT +)																
Diploid	27	0.1 ± 0.4	27	0	0	0	0	0	0	23	4	0	0	0	0	0
Near tetraploid	4	2.2 ± 2.9	4	0	0	0	0	0	0	2	0	0	1	0	0	1
Total	31															
50-5 cl 7																
PD 74 (hTERT +)																
Diploid	29	0.03 ± 0.18	29	0	0	0	0	0	0	28	1	0	0	0	0	0
Near tetraploid	4	0.5 ± 1	4	0	0	0	0	0	0	3	1	0	0	0	0	0
Total	33															
PD 125																
Diploid	20	0.6 ± 1.1	15	0	5[3]	0	0	0	0	18	2	0	0	0	0	0
50-5E																
PD 86																
Near Tetraploid	9	4.1 ± 1.3	0	2	3	4	0	0	0	**0**	**4**	**2**	**1**	**2**	**0**	**0**
PD 171																
Near Tetraploid	6	12.2 ± 3.3	0	0	0	0	2	1	3	**0**	**0**	**0**	**0**	**2**	**1**	**3**

[1]Consistent chromosomal findings are defined as an abnormality that is seen in 2 or more metaphase spreads; [2]Incidental chromosomal findings are defined as an abnormality that is seen in a single metaphase spread. The presence of multiple incidental findings shows cytogenetic heterogeneity and instability. The frequency distributions that are highlighted in bold show cell lines that have a significantly higher level of incidental chromosomal abnormalities when compared to the other cell lines; [3]A total of 5 metaphase spreads from the culture with 125 PD showed the same clonal abnormalities [der(X)t(5;4)(p13;p15);der(17)t(X;17)(?p11.2;p11.2)], suggesting that they all proliferated from a single abnormal cell.

these data were generated using in vitro translated p53 proteins rather than evaluating the functional activity of p53 in LFS human cells. Therefore, we have assessed the p53 functional status in the 50-5 cells (uninfected and stable hTERT-expressing) and spontaneously immortal 50-5E cells. Our approach involved determining both the conformation and transcriptional activation activity of p53 in the respective 50-5, 50-5-hTERT, and 50-5E cells. We initially performed an immunoprecipitation analysis using either wild type- or mutant-specific p53 monoclonal antibodies or a pantropic monoclonal antibody, followed by immunoblotting with a p53 polyclonal antibody. As shown, the 50-5 cells ectopically expressing telomerase, like the parental cells, expressed p53 in both wild type and mutant conformations, as previously reported in tumors from LFS patients,[34] with the level of mutant p53 protein appearing elevated relative to the wild type (Fig. 4A). The spontaneously immortal 50-5E cells also express both wild type and mutant p53 but at substantially lower levels than the 50-5 cells. A previous report indicating that the 50-5E cells express only barely detectable levels of p53 in the wild-type conformation,[12] likely is a consequence of using a lysis buffer containing the potent reducing agent, dithiothreitol.

In order to evaluate the functional status of p53, we assayed by Western blotting the levels of p53 and $p21^{waf-1}$ in the different 50-5 and 50-5E cultures following g-irradiation. In normal cells with wild type p53, DNA damage results in the accumulation of p53 and induction of $p21^{waf-1}$, a cyclin-dependent kinase inhibitor critical for triggering growth arrest.[35-36] Four hours after irradiation, we observed an accumulation of p53 and induction of $p21^{waf-1}$ in MCF-7 cells, a human breast cancer cell line with wild type p53, but not in the uninfected or telomerase-infected 50-5 cells or spontaneously immortal 50-5E cells (Fig. 4B). Complementary to our immunoprecipitation results (Fig. 4A), the 50-5E cells expressed substantially lower levels of p53 than the parental and hTERT-infected 50-5 cells and barely detectable levels of $p21^{waf-1}$ (Fig. 4B). Taken together, these data demonstrate that a methionine to threonine missense mutation at codon 133 blocks wild type p53 transcriptional transactivation activity in 50-5 (mortal and hTERT-expressing) throughout their lifespan, as well as in spontaneously immortal 50-5E cells. Moreover, since LFS cells ectopically expressing telomerase (and maintaining telomeres) have functionally inactive p53 yet stable karyotypes, it appears that telomere erosion is the primary driving force for the observed chromosomal instability.

DISCUSSION

In this study, we demonstrate that ectopic telomerase protects cancer prone human cells from chromosomal instability, a major factor influencing "classical" spontaneous immortalization and malignant transformation. Hackett et al.[37] have very recently reported that telomerase also prevents chromosomal instability in *S. cerevisiae*, hinting that this exogenous telomerase-mediated protective effect may be a more universal phenomena. Unlike previous studies involving ectopic telomerase in normal human cells,[9-10] LFS 50-5 cells harbor a missense mutation in the highly conserved DNA binding domain of p53,[12] which we and others[38] have shown results in functional inactivation of p53. It is generally believed that if p53 cannot respond to DNA damage by inducing apoptosis or senescence to irreversibly remove cells from the replicative pool or growth arrest to allow for repair, chromosomal aberrations will result. In this genomically unstable environment, LFS cells accumulate additional mutations and chromosomal rearrangements contributing to the increased frequency of neoplastic transformation but only in the presence of telomere shortening. Using a highly relevant human model system, we demonstrate that telomere dysfunction, rather than inactivation of p53, triggers the observed genomic instability.

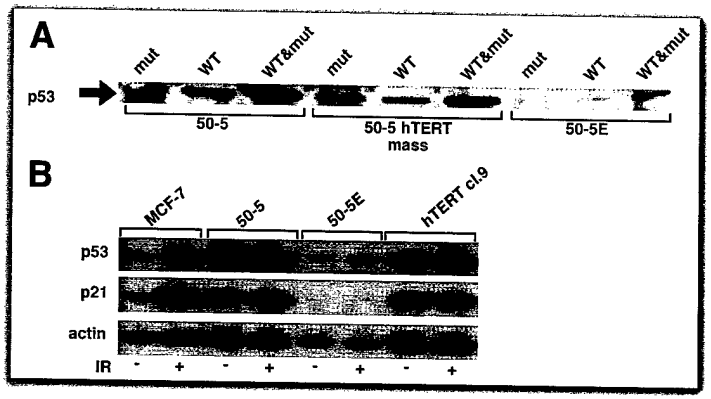

Figure 4. hTERT-infected 50-5 cells express wild type and mutant p53 protein but exhibit loss of p53 transactivation activity. (A) Immunoprecipitation analysis demonstrating that LFS cells express both wild type and mutant p53 protein; however, the spontaneously immortal 50-5E cells express substantially lower levels of both conformations than the uninfected and stably expressing hTERT cells. For each cell culture, 3×10^5 cell equivalents were immunoprecipated with Ab-3 (mutant-specific), Ab-5 (wild type-specific) or Ab-6 (pantropic) as described in "Materials and Methods". WT = wild type; mut = mutant. (B) Undetectable accumulation of p53 without induction of $p21^{waf-1}$ in parental or hTERT-infected 50-5 cells or spontaneously immortal 50-5E cells following γ-irradiation. The 50-5 cultures were treated with a single dose of γ-irradiation (10 Gy), lysed 4 hours later, and subjected to Western blotting. MCF-7 cells (a breast cancer cell line expressing wild type p53) served as a control for the DNA damage response, while actin served as a loading control. Although both 50-5E samples were conservatively underloaded, there appears to be disproportionately lower levels of expression of both p53 and $p21^{waf-1}$ proteins.

Inactivation of p53 facilitates the "classical" spontaneous immortalization. Boyle et al.[13-14] similarly report that human fibroblasts derived from patients with LFS containing a germline p53 mutation exhibit marked genomic instability in culture, but only as senescence approaches, suggesting that telomere erosion is driving the instability. Data from double telomerase/p53 knock-out mice further support a requirement for telomere shortening as a driving force of genetic instability, and the p53 deficiency serves to facilitate the process.[39-40] Despite highly analogous findings in the mouse studies, rather than preventing telomere shortening and crisis as we have done, telomeres slowly eroded through successive generations of $mTR^{-/-}$ $p53^{-/-}$ mice.

Based on well-founded data that telomeres stabilize and protect chromosomes,[41] ectopic telomerase likely protects against genomic instability by maintaining telomere lengths. However, it is possible that ectopic telomerase may also confer genomic stability in cells through chromosome healing or repair, as previously reported in other eukaryotes.[37,42-43] When chromosome breakage occurs in Tetrahymena, telomerase is able to recognize a nontelomeric sequence and then add telomeric tracts of DNA onto the broken ends. With this precedent established in lower eukaryotes, the ability of human telomerase to similarly remedy chromosomal breaks in vertebrates certainly warrants further investigation.

Our cells lack wild-type p53 function throughout their lifespan, yet it is not until they approach senescence (shorten telomeres) that the requisite chromosomal and genetic abnormalities arise that lead to the spontaneous, classically immortalized cells. p53 is inactivated at early passage, indicating p53 alone is incapable of inducing the chromosomal changes necessary for the spontaneous immortalization in the "classical" sense. Only after telomeres shorten to a critical length does the lack of p53 function play a key role in the immortalization process (we know from previous experiments that inactivation of wild-type p53 in normal mammary epithelial cells is absolutely required for the immortalization event [data not shown]). As a result, our data conclusively show that introduction of telomerase prevents spontaneous immortalization and the genetic and chromosomal instability associated with classical immortalization, providing the first experimental evidence that telomere erosion is the driving force for the genomic instability required for immortalization. Within a genomically unstable environment, we find that the lack of p53 function may assist in the immortalization process without being a driving force, consistent with data from mouse model systems.[40] However, our data in a human model system suggests that mutant p53 is only a facilitator of "classical" immortalization in cells with shortened telomeres.

Recently, Wang et al.[44] have suggested that telomerase expression in mammary epithelial cells results in activation of c-myc and potentially reactivation of endogenous telomerase. In our system, we have found no increase in c-myc levels after 150 PDs post-infection, nor does endogenous telomerase become reactivated (data not shown). Thus, we respectfully suggest that the cells used for the previous study spontaneously immortalized in the "classical" sense and that reduced levels of introduced hTERT were insufficient to maintain telomere lengths and genomic stability. However, when telomerase is expressed at substantial levels, as in our system presented here, telomere lengths are stabilized and genomic integrity is maintained without signs of "classical" immortalization.

Considering the wealth of data indicating that telomerase is essential for immortalization and continued cancer cell proliferation, it may seem paradoxical to conclude that telomerase protects against chromosomal instability and spontaneous immortalization. Reconciliation of this issue begins with an understanding that extended life-span cells via exogenous telomerase (i.e., normal or genomically-stable LFS cells) are biologically distinct from cells that have bypassed the senescence and crisis checkpoints ("classically" immortal), events in the cellular life-cycle that occur, in large part, due to telomere shortening.[11] A better understanding of how telomerase protects against immortalization could have implications for novel preventative measures for age-related, somatic malignancies, as well as for those malignancies that develop in association with inherited cancer susceptibility syndromes, including but not limited to Li Fraumeni Syndrome.

Acknowledgements

We thank Dr. David Gewirtz for critical reading of the manuscript, Xu Di for assistance with the $p21^{waf-1}$ Western blotting, and Catherine Rehder for assistance with the statistical analysis. Geron Corporation (Menlo Park) provided the hTERT cDNA and antibody. This work was supported by grant funding from Virginia's Commonwealth Health Research Board (SEH and CKJ-C), the Mary Kay Ash Charitable Foundation (SEH) and the American Cancer Society (LWE), and by Institutional support from the Department of Pathology and the Massey Cancer Center (LWE and SEH). SEH is a V Foundation Scholar.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 38, Issue of September 20, pp. 35509–35515, 2002
Printed in U.S.A.

Adriamycin-induced Senescence in Breast Tumor Cells Involves Functional p53 and Telomere Dysfunction*

Received for publication, June 3, 2002, and in revised form, July 1, 2002
Published, JBC Papers in Press, July 5, 2002, DOI 10.1074/jbc.M205477200

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Direct experimental evidence implicates telomere erosion as a primary cause of cellular senescence. Using a well characterized model system for breast cancer, we define here the molecular and cellular consequences of adriamycin treatment in breast tumor cells. Cells acutely exposed to adriamycin exhibited an increase in p53 activity, a decline in telomerase activity, and a dramatic increase in β-galactosidase, a marker of senescence. Inactivation of wild-type p53 resulted in a transition of the cellular response to adriamycin treatment from replicative senescence to delayed apoptosis, demonstrating that p53 plays an integral role in the fate of breast tumor cells treated with DNA-damaging agents. Stable introduction of hTERT, the catalytic protein component of telomerase, into MCF-7 cells caused an increase in telomerase activity and telomere length. Treatment of MCF-7-hTERT cells with adriamycin produced an identical senescence response as controls without signs of telomere shortening, indicating that the senescence after treatment is telomere length-independent. However, we found that exposure to adriamycin resulted in an overrepresentation of cytogenetic changes involving telomeres, showing an altered telomere state induced by adriamycin is probably a causal factor leading to the senescence phenotype. To our knowledge, these data are the first to demonstrate that the mechanism of adriamycin-induced senescence is dependent on both functional p53 and telomere dysfunction rather than overall shortening.

Most normal somatic cells continually shorten their telomeres after each cell division because of incomplete replication at the end of linear chromosomes (1, 2). The original hypothesis stated that when telomeres have become sufficiently shortened, replicative senescence is induced (3, 4). Tumor suppressor proteins such as p53 are required for this senescence arrest. Most cells with indefinite proliferative ability (*e.g.* human tumors and their derivative cell lines) express the enzyme telomerase to maintain telomeres, which allows for the continued cellular proliferation characteristic of human cancer (5, 6). Telomerase is a cellular reverse transcriptase containing two strictly required elements: a protein component, hTERT, and an RNA element, hTR (7–9). hTERT serves as the catalytic subunit, whereas hTR is utilized by hTERT as the template for catalyzing the addition of telomeric DNA to the end of the chromosome. The introduction of telomerase into normal human cells provides for telomere maintenance, prevention of senescence, and an extension of life span, indicating that gradual telomere shortening is one of the factors contributing to the onset of cellular senescence (10, 11). Recent evidence suggests that although telomere length is an important trigger for the onset of senescence, increased telomere dysfunction results in a loss of chromosome end protection and induction of the senescence state (12). These novel findings show that senescence can be induced without net telomere shortening, and that while length remains important, preservation of telomere integrity is critical regardless of telomere length.

The mechanism(s) of action of the anthracycline antibiotic adriamycin, a drug that has long been a mainstay in the treatment of breast cancer (13), have been studied extensively (14). Adriamycin promotes apoptotic cell death in a variety of experimental tumor cell lines (15–18). However, we have demonstrated that MCF-7 breast tumor cells fail to undergo a primary apoptotic response after either acute or chronic exposure to adriamycin (19, 20). Here, we show that the growth-arrested state associated with acute adriamycin treatment of MCF-7 cells (20) results in down-regulation of telomerase activity and induction of a senescence phenotype. MCF-7 cells expressing the human papillomavirus type 16 (HPV-16)[1] E6 protein show degradation of p53, a lack of overall p53 function, and conversion from a senescent phenotype to apoptosis after adriamycin treatment, demonstrating that p53 is critical for replicative senescence in drug-treated MCF-7 cells. Exogenous expression of hTERT provides for increased telomerase activity and elongated telomere lengths but does not protect MCF-7 cells from drug-induced cellular senescence. We find that adriamycin-induced DNA damage appears to preferentially target chromosome ends resulting in substantial telomere-related cytogenetic abnormalities, indicating that the observed senescence is because of telomere dysfunction rather than overall shortening. Our data clearly indicate that the senescence program

* This work was supported in part by the Mary Kay Ash Charitable Foundation (to L. W. E. and S. E. H.), the Department of Defense Breast Cancer Research Program Grant DAMD 17-01-0441 (to D. A. G. and S. E. H.), Virginia's Commonwealth Health Research Board (to C. K. J.-C.), and NCI, National Institutes of Health Postdoctoral Training Grant CA 85159-01 (to P. A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HPV, human papillomavirus; PBS, phosphate-buffered saline; TRAP, telomeric repeat amplification protocol; TRF, terminal restriction fragment; TUNEL, Tdt-mediated dNTP nick end labeling; kb, kilobase(s); MAPK, mitogen-activated protein kinase.

observed in adriamycin-treated MCF-7 cells requires functional p53 and telomere dysfunction.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium and trypsin-EDTA (0.5% trypsin, 5.3 mM EDTA) were obtained from Invitrogen. L-Glutamine, penicillin/streptomycin (10,000 units penicillin/ml and streptomycin 10 mg/ml), and fetal bovine serum were obtained from Whittaker BioProducts (Walkersville, MD). Adriamycin and retinoic acid were purchased from Sigma, reconstituted in molecular biology grade water, and stored as aliquots at −20 °C until dilution in culture medium immediately before cell treatments.

Cell Culture and Adriamycin Treatment—The MCF-7 and MDA-MB231 breast tumor cell lines were obtained from the NCI Frederick Cancer Research Facility. Cells were maintained as monolayers in RPMI 1640 medium supplemented with glutamine (0.292 mg/ml), penicillin/streptomycin (0.5 ml/100 ml medium), and 10% fetal bovine serum. All cells were cultured at 37 °C in 5% CO_2 and 100% humidity. Twenty-four hours after plating, cells were exposed to 1 μM adriamycin for 2 h, rinsed once in PBS, and then maintained in supplemented RPMI 1640 medium. The retroviral packaging cells, PA317 HPV-16 E6 and PA317 hTERT, and their vector controls (pLXSN and pBABEpuro, respectively) were obtained from Dr. Jerry W. Shay (UT Southwestern, Dallas, TX) and were maintained in Dulbecco's modified Eagle's medium with 10% bovine calf serum as described above. Target MCF-7 cells were infected and selected using standard procedures as described previously (21).

Telomerase Activity Assay—Telomerase activity was determined by the telomeric repeat amplification protocol (TRAP) using the TRAPeze kit (Intergen, Purchase, NY) as described previously (6, 22). The reactions were extended for 30 min at 30 °C, and extension products were PCR-amplified for 27 cycles and resolved on a 10% polyacrylamide gel. The gel was then exposed to a PhosphorImaging cassette (Amersham Biosciences) and directly scanned and analyzed using ImageQuant Software (Amersham Biosciences). A positive result indicating telomerase activity was shown by the presence of a 6-bp progressive ladder. A 36-bp internal standard verified successful amplification and served as a useful standard for relative quantitation. Telomerase activity was semi-quantitatively calculated using the ratio of the intensity of the telomerase ladder to the intensity of the 36-bp internal standard.

Reverse Transcription-PCR Analysis of Endogenous Versus Exogenous hTERT Expression—Total RNA was isolated from cells in logarithmic growth phase using TRIzol (Invitrogen) as recommended by the manufacturer. According to the manufacturer's established protocol, 3 μg of total RNA from each cell culture were reverse-transcribed in a 20-μl reaction volume using decamers and the regular reaction buffer provided in the first strand synthesis kit, RETROscript (Ambion Inc., Austin, TX). A 2.5-μl aliquot of cDNA was used for PCR amplifications. hTERT was amplified using the oligonucleotide primer hTERT (5′-GACTCGACACCGTGTTCACCTAC-3′) paired with either Endo 1 (5′-ACGTAGAGCCCGGCGTGACAG-3′) or pBABE (5′-GACACACATTC-CACAGGTCG-3′) (23), which selectively amplify endogenous or exogenous hTERT, respectively. For both primer pairs, the thermocycling conditions were: 94 °C for 5 min followed by 34 cycles of 94 °C for 45 s, 62 °C for 30 s, and 72 °C for 45 s. Amplification of 18 S RNA was performed as a control with 18 S PCR primer pairs (Ambion, Inc.) using the same thermocycling conditions described above with only 22 cycles completed. Amplified products (exogenous hTERT: 175-bp; endogenous hTERT: 219-bp; 18 S: 488-bp) were resolved on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Telomere Length Analysis—Telomere length was determined using terminal restriction fragment (TRF) analysis as noted before (10, 21). DNA was isolated from cells using genomic tips (Qiagen, Santa Clarita, CA) digested with a mixture of *Hin*fI, *Alu*I, and *Rsa*I restriction enzymes (Invitrogen) and resolved on a 0.7% agarose gel. The DNA ladder and G-rich telomere probe $(TTAGGG)_4$ were labeled with [γ-^{32}P]ATP (6000 Ci/mmol) with unincorporated label removed from the reaction using the QIAquick nucleotide removal kit (Qiagen). The dried agarose gel was subjected to in-gel hybridization with the labeled probe, washed repeatedly with varying concentrations of SSC buffer, and exposed to a PhosphorImaging cassette for 2–24 h. An estimation of median telomere length for comparison purposes only was made by measuring the radioactive smear, taking the midpoint of its length for characterization of isogenic cell lines. A more rigorous determination of average telomere length was accomplished using an established protocol as described previously (24).

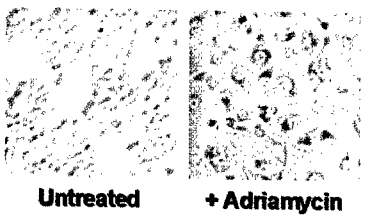

FIG. 1. **Expression of senescence-associated β-galactosidase in MCF-7 cells following acute exposure to adriamycin.** Cells were exposed to 1 μM adriamycin for 2 h, and β-galactosidase expression was assessed 4 days after adriamycin treatment. Shown are representative microscopic fields from untreated (*left panel*) and adriamycin-treated (*right panel*) cells. Original magnification for both is ×20. Note the substantial increase in cellular volume and the blue staining of the treated (senescent) cells.

β-Galactosidase Histochemical Staining—MCF-7 cells were washed twice with PBS and fixed with 2% formaldehyde, 0.2% glutaraldehyde for 5 min. The cells were then washed again with PBS and stained with a solution of 1 mg/ml 5-bromo-4-chloro-3-inolyl-β-galactosidase in dimethylformamide (20 mg/ml stock), 5 mM potassium ferrocyanide, 150 mM NaCl, 40 mM citric acid/sodium phosphate, pH 6.0, and 2 mM $MgCl_2$ as described previously (10, 25). Following overnight incubation at 37 °C, the cells were washed twice with PBS, and the percentage of positively stained cells was determined after counting three random fields of 100 cells each. As a positive control for β-galactosidase expression, MCF-7 cells were exposed to 200 nM retinoic acid for 4 days and then stained. Representative microscopic fields were photographed under a ×20 objective.

Western Analysis for p53 and $p21^{waf-1}$ Proteins—MCF-7 cells (parental, HPV-16 E6, and pLXSN) were treated with 1 μM adriamycin for 2 h, washed with PBS, and then cultured for an additional 2 h prior to lysing in a standard radioimmune precipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 100 mM dithiothreitol, and protease inhibitors). The respective isogenic untreated MCF-7 cultures were included for assessing the constitutive levels of p53 and $p21^{waf1}$. Protein concentrations were determined using a Lowry-based spectrophormetric assay (Bio-Rad) according to the manufacturer's protocol. A 15-μg aliquot of each sample was separated by SDS-PAGE and electrotransferred onto nitrocellulose membrane. A standard blotting protocol was then performed using pantropic p53 (1:500 of Ab-6, Oncogene Research Products) and a $p21^{waf-1}$ monoclonal (1:500, Signal Transduction Laboratories) antibodies followed by peroxidase-conjugated anti-mouse IgG (1:10,000, Amersham Biosciences). A chemiluminescent reaction (ECL Reagent, Amersham) was used for detection. To control for protein loading, the membrane was subsequently probed with an actin antibody (1:5000, Sigma) and processed as described above.

TUNEL Assay—MDA-MB231, MCF-7, and MCF-7-E6 cells were directly seeded onto 4-well chamber slides, treated with 1 μM adriamycin for 2 h, and then after 5 days, stained by TUNEL (Roche Molecular Biochemicals) according to the manufacturer's instructions. Cells were counterstained with DAPI (Sigma) to allow easy determination of the total number of cells per field. Three random fields of at least 300 cells in monolayer culture were scored to determine the percentage of cells undergoing apoptosis. Values represent the mean ± S.D.

Cytogenetic Analysis—Metaphase chromosomes were harvested from the MCF-7 cell cultures using standard methods (26). Actively dividing cells were blocked in mitosis with 0.1 μg/ml colcemid for 2 h, incubated in 0.075 M KCl hypotonic solution for 20 min, and fixed in methanol: glacial acetic acid (3:1). Slides were made using standard procedures, and metaphase chromosomes were visualized using conventional Geimsa staining (27). Metaphase spreads were scored for chromosomal findings from both the MCF-7-hTERT cell cultures before and after adriamycin treatment. A total of 200 metaphase spreads (100 each) were evaluated (28). The frequency of the types of chromosomal abnormalities seen in the cells with and without adriamycin treatment were compared using a contingency chi-square test with an $\alpha < 0.05$ significance level.

RESULTS

Acute Adriamycin Treatment Induces Senescence and Represses Telomerase Activity—In this study, our initial objective was to determine whether MCF-7 cells undergo replica-

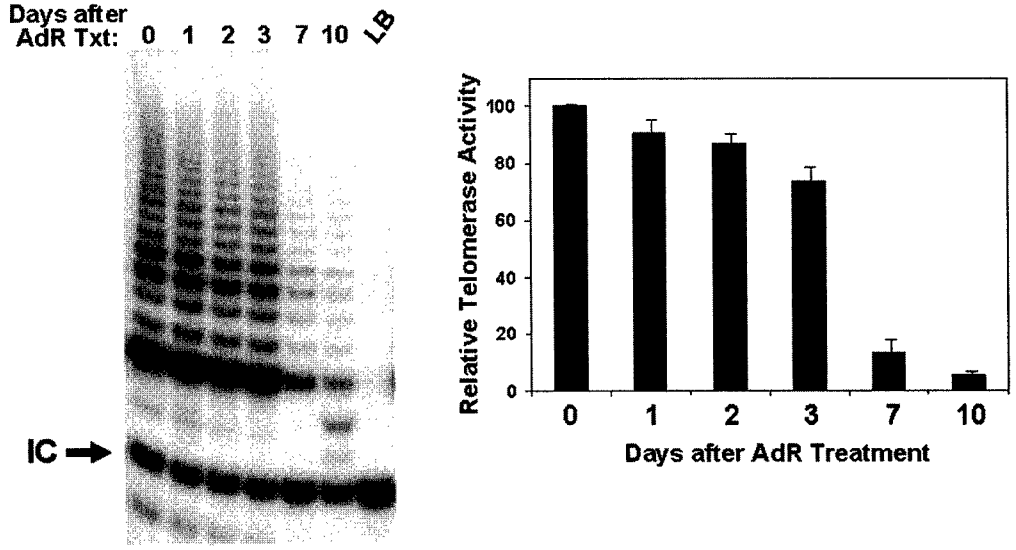

FIG. 2. **Influence of adriamycin on telomerase activity in the MCF-7 breast tumor cell line.** *Left panel*, cells were exposed to 1 μM adriamycin for 2 h and then assayed for telomerase activity using the TRAP assay (6, 22). One thousand cell equivalents were used for each reaction with a representative experiment shown. *IC* denotes the 36-bp internal control band that serves to normalize sample to sample variation. Nonspecific banding between the 36-bp IC and the initial 50-bp telomerase band (seen in the 10-day sample) is unrelated to telomerase activity and is not included in the quantitation. *Right panel*, relative quantitation of telomerase activity levels after adriamycin treatment of MCF-7 cells using the 0 time point as 100%. *LB*, lysis buffer; *AdR*, adriamycin.

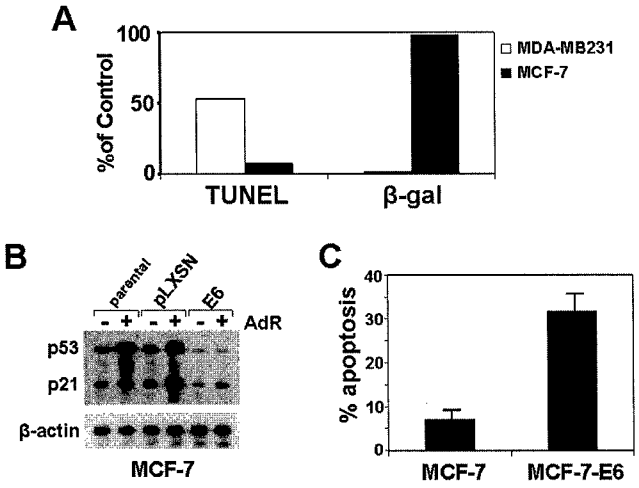

FIG. 3. **Functional p53 is required for induction of senescence after adriamycin treatment.** *A*, comparison of breast tumor cells with differing p53 status, MCF-7 (wild-type p53) and MDA-MB231 (mutant p53), for response to adriamycin treatment. Five days post-treatment, cells were assessed for senescence as indicated by β-galactosidase induction and for apoptosis using the TUNEL assay. Three independent fields of stained cells were counted, and the average percent positivity compared with untreated controls (% of control) was calculated. *B* and *C*, MCF-7-derived cells were treated with 1 μM adriamycin for 2 h and tested for p53 and $p21^{waf-1}$ induction (*B*) and induction of apoptosis (*C*). *B*, parental MCF-7 (*parental*), MCF-7 with vector pLXSN only, and MCF-7 infected with HPV-16 E6 (*E6*) were treated and harvested 2 h after treatment with adriamycin (+) and compared with untreated controls (−). Cells were extracted, and 15 μg of total protein were electrophoresed (12% SDS-PAGE). Transferred blots were probed using antibodies specific to p53, $p21^{waf-1}$, and β-actin followed by detection using chemiluminescence. The modest increase in $p21^{waf-1}$ levels observed in the MCF-7-E6 cells after adriamycin treatment is within the range of experimental error, possibly representing a p53-independent increase in $p21^{waf-1}$. *C*, quantitation of TUNEL positivity in the MCF-7 and MCF-7-E6 cells at 5 days post-treatment. Values represent the mean ± S.D. based on three random fields with 300 cell counts/field.

tive senescence following acute exposure to 1 μM adriamycin. β-Galactosidase expression, a marker of cellular senescence (10, 25), was present in the MCF-7 cells as early as 2 days after acute exposure to adriamycin (data not shown). After 1 week, histochemical staining was intense and expressed in virtually every cell of the culture (Fig. 1). Cells expressing this senescence marker were typically much larger in size and multinucleated, both of which are morphological features indicative of a senescent state. Continuous exposure to retinoic acid used as a positive control (29) induced β-galactosidase activity in MCF-7 breast tumor cells as expected (data not shown). Because one could argue that β-galactosidase staining in this system may only reflect a lack of cell division rather than true senescence, MCF-7 cells were held in a non-dividing state by serum removal for 7 days and stained for β-galactosidase. In contrast to the adriamycin-treated cells, β-galactosidase expression was detected only very rarely in non-dividing MCF-7 cells, consistent with that observed for untreated MCF-7 cells (0.3% + 0.2).

Because induction of senescence has been closely associated with the suppression of telomerase activity (30, 31), we also assessed the influence of acute adriamycin treatment on telomerase activity in the MCF-7 cells. Adriamycin produced a time-dependent decline of telomerase activity in MCF-7 cells (Fig. 2) that differs from the recently described early daunorubicin-mediated telomerase inhibition in lung cancer cells (85% reduction at 24 h) (32). Although the effects of adriamycin on telomerase activity were not significant within the first 1–3 days of drug exposure, exhibiting a half-life consistent with previous results (30, 31), telomerase activity was reduced 90% after 7 days with a greater than 95% reduction after 10 days (Fig. 2*B*).

Inactivation of p53 Results in Induction of Apoptosis Rather Than Senescence After Adriamycin Treatment—Because MCF-7 cells express wild-type p53 but lack functional caspase 3 protein (33), the induction of replicative senescence during adriamycin treatment may be attributed to a combination of p53-mediated senescence (34) and the inability to progress down the apoptotic pathway. We tested this hypothesis using another p53-positive breast tumor cell line with functional caspase 3 (ZR-75) and found that adriamycin treatment results in an identical senescence pattern without detectable apoptosis (data not shown), consistent with that observed for the MCF-7

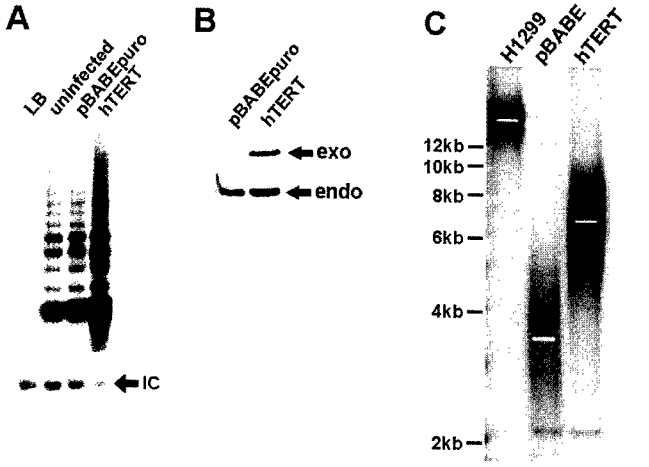

FIG. 4. **Expression of hTERT in MCF-7 cells elevates telomerase activity and elongates telomeres.** MCF-7 cells were retrovirally infected with hTERT, and a stable population of cells was selected with puromycin. *A*, a representative TRAP assay with MCF-7 parental (*uninfected*), vector only (*pBABEpuro*), and hTERT-selected populations at 1000 cells/reaction. A lysis buffer (*LB*) only sample served as a negative control. IC denotes the 36-bp internal control band. Two independent experiments yielded elevated activity of ~5–7-fold compared with parental or vector controls. *B*, Reverse Transcription-PCR for hTERT was accomplished using primers specific for endogenous (*endo*, 219-bp, present in both pBABEpuro and hTERT cells) and exogenous (*exo*, 175-bp, present only in the hTERT cells) hTERT as described under "Experimental Procedures." Levels of 18-s amplification were indistinguishable between the four samples (data not shown). *C*, TRF analysis to assess telomere sizes in vector controls (pBABEpuro) and hTERT MCF-7 cells. H1299 is a lung adenocarcinoma cell line that serves as a positive control. *White bars* indicate the median size of telomere length. The *numbers* on the *left* show the positions of a DNA-sizing ladder (in kb).

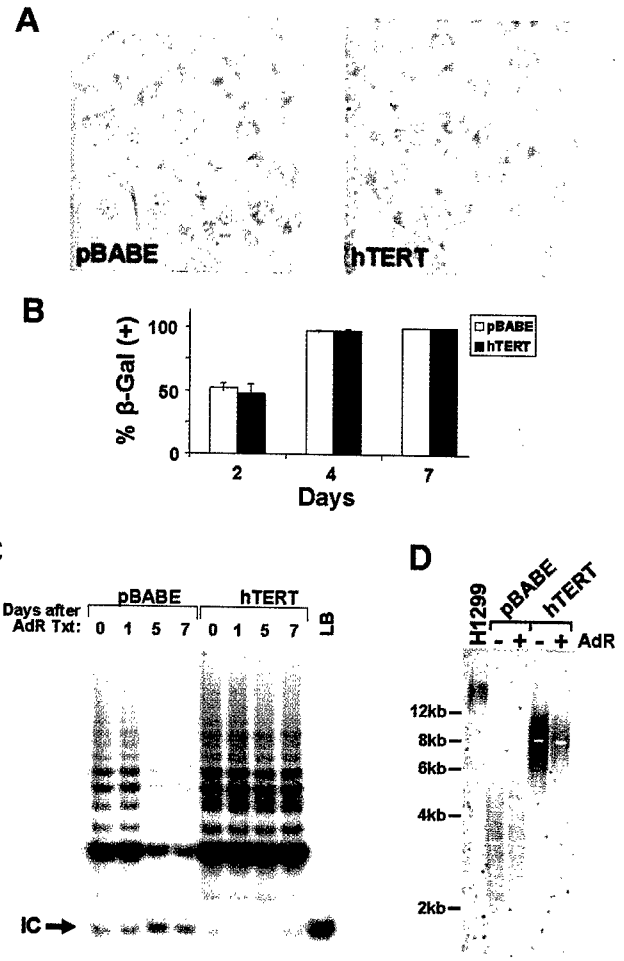

FIG. 5. **Adriamycin-induced senescence in MCF-7 and MCF-7-hTERT cells is independent of telomere length.** *A*, MCF-7-pBABEpuro and MCF-7-hTERT cells were histochemically stained for β-galactosidase expression at day 7 after treatment. Original magnification, ×20. *B*, quantitation of β-galactosidase expression in the MCF-7-pBABEpuro and MCF-7-hTERT cells over time. Values represent the mean ± S.D. based on three random field with 100 cell counts/field. *C*, MCF-7-pBABEpuro and MCF-7-hTERT cells were acutely treated with adriamycin (1 μM), harvested at the indicated days, and analyzed by the TRAP assay using 500 cell equivalents/sample. *IC* denotes the 36-bp internal control band. *D*, TRF analysis of MCF-7-pBABEpuro and MCF-7-hTERT cells before and 72 h after acute adriamycin treatment. The *numbers* on the *left* show the positions of a radiolabeled DNA marker in kb, and the *white bars* indicate the position of the measured mean telomere length (24). *AdR*, adriamycin.

cells (quantitation in Fig. 3*A*). In addition, adriamycin-treated breast tumor cells with mutant p53 (MDA-MB231) exhibit a delayed apoptosis rather than senescence (Fig. 3*A*). These observations taken together suggested a pivotal role for p53 in the induction of senescence in breast tumor cells and that inactivation of p53 in MCF-7 cells may allow conversion to an apoptotic pathway following DNA damage (35). To test this hypothesis, MCF-7 cells were infected with HPV-16 E6, which resulted in the degradation and inactivation of p53 as denoted by the lack of p53-mediated transcriptional activation of its downstream target $p21^{waf-1}$ (Fig. 3*B*) after drug treatment. Even though we observe a slight increase (2–3-fold) in $p21^{waf-1}$ independent of p53, acute treatment of MCF-7-E6 cells with 1 μM adriamycin resulted in a delayed apoptotic event rather than senescence 5 days post-treatment using the TUNEL assay (quantification shown in Fig. 3*C*). Collectively, these data suggest that wild-type p53 activity is necessary for the induction of the replicative senescence phenotype observed in the adriamycin-treated MCF-7 cells.

Ectopic Expression of hTERT in MCF-7 Cells Results in Increased Telomerase Activity and Telomere Length—To assess the involvement of telomerase and telomere length in the adriamycin-induced senescence response, we retrovirally infected the hTERT gene into MCF-7 cells and selected for stable integration using puromycin. The introduction of hTERT into MCF-7 cells resulted in elevated telomerase activity (nearly 5-fold) (Fig. 4*A*), continuous expression of exogenous hTERT (Fig. 4*B*), and a substantial increase in telomere length from a median length of 3.5 to 7 kb (Fig. 4*C*). Because MCF-7 cells already have a substantial amount of telomerase activity, it was possible that hTERT expression would not result in an increase in telomerase activity or telomere elongation. However, our data clearly show that hTERT is the limiting factor for telomerase elevation and telomere elongation in MCF-7 cells.

Senescence in Adriamycin-treated MCF-7 Cells Is Induced by Telomere Dysfunction but Not Telomere Shortening—We hypothesized that because telomere length has been shown to be a primary cause of cellular senescence in normal cells (10, 11), elongation of telomeres using hTERT would prevent the induction of the senescence pathway in adriamycin-treated breast cancer cells or, at a minimum, postpone it. Both MCF-7-pBABEpuro cells and MCF-7-hTERT cells with elongated telomeres senesced with the same frequency and timing (Fig. 5, *A* and *B*) nearly identical to uninfected MCF-7 controls. As expected, adriamycin treatment of MCF-7-hTERT cells did not result in a decline in telomerase activity levels (Fig. 5*C*), whereas vector-only (pBABE) controls exhibited a decline in activity similar to uninfected MCF-7 cells (compare with Fig. 1).

Because there was no lag in the timing of the onset of senescence in the MCF-7-hTERT cells, overall telomere short-

TABLE I
Characterization of cytogenetic abnormalities in adriamycin-treated MCF-7 cells

Cell line	Metaphases scored	Metaphases with abnormalities[a]	Structural anomalies involving chromosome ends				Dicentrics[b]	Breaks involving interstitial sites	
			End fusions	Rings	Radials	End breaks		Chromatid	Chromosome
MCF-7 hTERT	100	9	3	1	0	0	3	2	0
MCF-7 hTERT + AdR[c]	100	72	55	3	6	13	5	22	2

[a] Several spreads had more than one structural change, so the total number of anomalies seen is greater than the number of abnormal spreads.
[b] The end *versus* interstitial nature of the breaks giving rise to dicentric chromosomes could not always be clearly delineated. Thus, these aberrations are not categorized as a structural change clearly arising from an end compared to an interstitial region of the chromosome.
[c] AdR, adriamycin.

ening does not appear to be involved in the senescence process. To show this conclusively, at 24 (data not shown) and 72 h when the majority of cells are β-galactosidase-positive after acute treatment with adriamycin, changes in telomere length were determined for treated and untreated cells (representative TRF shown in Fig. 5*D*). Mean telomere length calculations (24) provide a quantitative estimate of changes in telomere length after treatment: MCF-7-pBABE (untreated 3.4 kb and treated 3.4 kb) and MCF-7-hTERT (untreated 8.3 kb and treated 8.1 kb). The 200-bp difference in calculated average telomere length in the MCF-7-hTERT cells is not significantly different and is within the range of experimental error. Thus, we found no substantial change in telomere length in treated and untreated MCF-7 and MCF-7-hTERT cells, demonstrating that the senescence phenotype observed in adriamycin-treated cells is not directly related to overall telomere shortening.

Because recent evidence implicates telomere dysfunction as a cause of replicative senescence (12), we determined the types of chromosomal changes induced by adriamycin treatment. To eliminate the possible contributions of shortened telomeres, we used the MCF-7-hTERT cells and evaluated the frequency and location of structural chromosomal changes in MCF-7-hTERT cells. As expected, adriamycin induced many structural anomalies related to chromosomal ends such as end:end fusions, end breaks, and radial chromosomes (Table I). Interestingly, the number of telomere-associated breaks and rearrangements (77 total observations excluding dicentrics) were overrepresented compared with interstitial breaks (24 observations) ($p \leq 0.0001$). These data suggest that adriamycin, a potent topoisomerase II inhibitor, preferentially produced breaks in distal chromosomal sequences and that these telomeric changes may ultimately contribute to the onset of replicative senescence in p53 wild-type breast tumor cells.

DISCUSSION

Replicative senescence in normal human cells involves the action of the tumor suppressors p53 and pRB, presumably in response to the DNA damage signal elicited by shortened telomeres and/or telomere dysfunction. We have previously demonstrated prolonged growth arrest and the absence of an initial apoptotic response after exposure of MCF-7 breast cancer cells to either chronic treatment of a sublethal (50 nM) concentration of adriamycin (19) or a single acute dose (1 μM) for 2–4 h (20). Others have recently reported (29, 36) that chronic exposure to sublethal concentrations of adriamycin promotes senescence in a variety of solid tumor cell lines based on β-galactosidase expression and cell morphology. Here, we clearly demonstrate that acute treatment of breast tumor cells with a clinically relevant dose of adriamycin (1 μM) results in the induction of replicative senescence and down-regulation of telomerase activity.

To determine whether telomere shortening was the ultimate cause of the observed senescence following acute adriamycin exposure, we generated an isogenic strain of MCF-7 with elongated telomeres. The catalytic subunit of telomerase, hTERT, was over-expressed in MCF-7 cells, which provided for increased telomerase activity and elongated telomere lengths. Upon treatment with adriamycin, the MCF-7-hTERT cells underwent senescence with identical frequency and periodicity as the parental or vector-only controls. These data clearly indicate that constitutive expression of telomerase does not delay or prevent the senescence program from occurring, suggesting a telomere length-independent cause for the senescence growth arrest in treated breast tumor cells. In addition, because adriamycin-treated MCF-7-hTERT cells continue to express telomerase activity even after treatment and growth arrest, the suppression of telomerase activity in parental MCF-7 cells is probably transcriptional rather than through proteolysis. In fact, our preliminary data suggest that endogenous hTERT mRNA levels are reduced within the first 24 h after treatment.[2]

We also found no detectable shortening in telomere lengths after drug treatment in either the control cells with short telomeres or the MCF-7-hTERT cells with elongated telomeres. These results provide direct experimental evidence that the senescence observed in adriamycin-treated MCF-7 cells is not telomere length-based, a result consistent with the inability of exogenous telomerase to prevent the premature senescence that occurs in normal cells strains after overexpression of oncogenic Ha-Ras (37). It is possible that an individual chromosome within the cell has shortened telomeres after treatment because of chromosome breaks at the telomere (38), which is beyond the limits of detection for our assay. However, given that virtually all of the MCF-7-pBABEpuro and MCF-7-hTERT cells senesced within the identical time frame, it is unlikely that this drug-induced senescence is the result of telomere shortening. The elongated telomeres would require significantly more population doublings to shorten telomeres enough to induce a telomere length-based senescence.

Instead, we propose that the senescence induced in these solid tumor-derived cell lines by adriamycin is in fact attributed to a reduction in the protective function of the chromosome ends (*i.e.* telomere dysfunction). As a result of adriamycin treatment, chromosomal ends were preferentially targeted for DNA damage, presumably induced by deregulation of topoisomerase II, where single strand and double strand breaks accumulate and force deprotection at the telomere. Consistent with previous findings for normal cell cultures with genetic manipulation (12), we find karyotypic instability as a result of telomere dysfunction and a lack of chromosome end protection after chemotherapeutic treatment. Our data clearly show that adriamycin-induced cytogenetic abnormalities at chromosome ends were significantly elevated compared with interstitial changes. Furthermore, one can speculate that the mechanism of the adriamycin-induced telomere-related abnormalities is probably the result of deregulation of telomere binding proteins and disruption of the telomere loop structure (39). Direct proof of this hypothesis is currently under investigation.

[2] L. W. Elmore, D. A. Gewirtz, and S. E. Holt, unpublished data.

The absence of an initial apoptotic response to adriamycin in the MCF-7 breast tumor cells may reflect the generalized refractoriness of breast tumor cells to apoptosis induced by DNA damage. One of the general criticisms with using the MCF-7 breast tumor cell line as a model is its lack of functional caspase 3 expression (33), reasoning that in response to adriamycin, these cells are unable to undergo apoptosis and that because they express wild-type p53, senescence is induced. Our work and that of others demonstrate an essentially identical pattern of response to adriamycin (initial non-apoptotic cell death followed by prolonged growth arrest) in a variety of p53-positive breast tumor cells including MCF-7 and ZR-75–1 cells (data not shown) (33). We also find that MCF-7 cells ectopically expressing caspase 3 undergo drug-induced senescence comparable to parental controls.[3] Preliminary studies have demonstrated that adriamycin induces MAPK and promotes the phosphorylation of the BAD protein, both of which may confer protection against apoptosis in breast tumor cells. It is possible that cells responding to adriamycin through a senescence arrest rather than apoptosis may ultimately die through another process such as reproductive cell death (29).

Because p53 is intimately involved in the senescence process in primary normal cell strains (34), we sought to define its role in the adriamycin-induced senescence phenotype in MCF-7 cells. We found that non-isogenic cell lines with mutant p53 (MDA-MB231) exhibited very little senescence after treatment with adriamycin but instead displayed a delayed apoptosis effect. To isogenically determine whether p53 was critical in the difference between senescence and apoptosis induction, MCF-7 cells were generated, which exogenously expressed the HPV-16 E6 oncogene to eliminate p53. Introduction of HPV-16 E6 resulted in the degradation and inactivation of the p53 protein, abolishing a sustained p53-mediated DNA damage response when treated with adriamycin. Yet, instead of undergoing senescence as before, treated MCF-7-E6 cells underwent a delayed programmed cell death, similar to other breast tumor cell lines without functional p53 (MDA-MB231). Seemingly inconsistent with our findings, a previous report concludes that MCF-7 cells with disrupted p53 (MCF-7-E6) are not sensitized to adriamycin (40). However, unlike our study that employed a comparative quantitative assessment of the frequency of apoptosis and senescence in isogenic cell lines, these investigators utilized clonogenic survival as their end point assay, which is a more limited approach that would not distinguish between a senescent or apoptotic cell.

Although it is clear that E6 has a number of functions unrelated to p53 inactivation, the currently defined role for HPV-16 E6 is mainly associated with the prevention of apoptosis during transformation (reviewed in Ref. 41). Thus, although it is formally possible that E6 is partially responsible for the shift of MCF-7 cells from senescence to apoptosis after adriamycin treatment, it is more probable that the elimination of p53 function is the mechanism for the conversion. This conclusion is supported by the recent data on p53 and $p16^{INK4a}$ in murine tumors that indicates p53 status is critical in determining drug-induced cellular fate (42). Although they find that p53 and $p16^{INK4a}$ play an equally important role in murine tumors, MCF-7 cells treated with adriamycin require wild-type p53 to undergo senescence in the absence of $p16^{INK4a}$, suggesting that adriamycin-induced senescence in breast tumor cells does not call for $p16^{INK4a}$. Taken together, our results indicate the following: 1) Programmed cell death after adriamycin treatment is possible in the absence of functional caspase 3; 2) Functional p53 is critical for the senescence phenotype observed in MCF-7 cells after adriamycin treatment. Our experimental data suggest that breast tumors with a loss of wild-type p53 function would be significantly more sensitive to adriamycin-induced apoptosis. Thus, assessing the p53 status in clinical specimens may have value for tailoring chemotherapeutic treatments for breast cancer patients, especially those involving adriamycin.

[3] L. W. Elmore and S. E. Holt, unpublished observations.

Acknowledgment—We thank Melissa Landon for critical experimental assistance.

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